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13. ABSTRACT (Maximum 200 Words) We have studied three prostate cancer lines for their ability to make proteins that alter the growth of cells. These proteins, called "SIRPs" (signal regulatory proteins), are expressed on the surface of cells. There are two types of SIRPs. One inhibits cell growth, while the other enhances it. SIRPs also interact with proteins expressed on cells belonging to the immune system. Thus, SIRPs may regulate both the growth of prostate cancer cells and their interactions with the immune system. We first showed that prostate cancer cells express a gene for an inhibitory SIRP. We have now made new reagents (monoclonal antibodies), which demonstrate the expression of SIRP proteins on at least two of the three prostate cancers. By gene transfer, we have also expressed an inhibitory SIRP at high levels in one of the prostate cancer cell lines. This will allow us to study the effect of the SIRP on the response of the cancer cells to growth hormones, and to examine the effects of mutations in SIRPs. We are also defining the range of SIRP genes expressed in the prostate cancer cells. These were the goals of our proposal, and we expect to accomplish them all.		
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INTRODUCTION

Our studies are based on our identification by PCR of transcripts for signal regulatory protein $\alpha 1$ (SIRP $\alpha 1$) in three prostate cancer cell lines, PC-3, LNCaP, and DU-145. We proposed six objectives:

1. Examine SIRP transcript size and expression level in different prostate cancer cell lines by Northern blotting.
2. Use RT-PCR to obtain and sequence full-length SIRP transcripts from the prostate cancer cell lines PC-3, DU-145, and LNCaP.
3. Use hybridization to screen cDNA libraries from these prostate cancer cell lines to identify additional SIRP cDNA clones.
4. Overexpress wild-type and mutated SIRPs in prostate cancer lines and, as a control, in NIH3T3 fibroblasts, to assess their effect on the cellular growth response to EGF. These will include studies of the effect of cross-linking SIRPs on cell growth. Mutational studies will include: for alpha SIRPs, mutate the cytoplasmic tyrosine to phenylalanine, for beta SIRPs, mutate the transmembrane lysine to alanine.
5. Assess the effect of EGF on the phosphorylation of SIRPs in prostate cancer cells and of associated proteins obtained by co-immunoprecipitation.
6. Produce monoclonal antibodies (mAbs) against SIRPs.

BODY

During the first year, we have made substantial progress toward our objectives, particularly Objective 6, the production of mAbs against SIRPs. To generate anti-SIRP monoclonal antibodies, the cDNAs for SIRP $\alpha 1$ and for SIRP $\beta 1$ were first 'tagged' by placing a known epitope (FLAG) at the N-terminus, by use of recombinant cDNA. Antibody to FLAG was then used to identify expression of SIRPs on CHO cells transfected with these constructs. Stable transfectants were used to immunize BALB/c mice, from which spleen cells were obtained and fused with Sp2/0 cells. The supernatants were screened for binding to 293T cells transiently transfected with the same construct. Binding to FLAG was excluded by screening against FLAG expressed on a different protein, and binding to other determinants on CHO was excluded by staining untransfected cells (Figure 1, APPENDIX 1).

SIRP $\alpha 1$ and SIRP $\beta 1$ are highly homologous, and our two monoclonal antibodies raised against SIRP $\alpha 1$ recognize both SIRP $\alpha 1$ and SIRP $\beta 1$, as assessed by staining of cells transfected with either receptor. Although these antibodies thus do not define the type of SIRP, staining of prostate cancer cells with either of the two antibodies supports the expression of SIRPs on PC-3 and, to a lesser extent, on DU-145 (Fig. 2, APPENDIX 1). SIRPs could not be detected on LNCaP cells (Fig. 2). These findings correlate with the intensity of RT-PCR product generated by SIRP primers, using RNA from each cell line as template, i.e., the greatest amount of product was from PC-3 cells, while DU-145 cells produced less, and LNCaP cells produced only a low-intensity band. Expression of SIRPs on prostate cancer cells was also confirmed by a previously published mAb, IL-A24, which was raised against bovine SIRP (MyD1). This antibody cross-reacts with human SIRPs. These findings have been accepted for presentation at the Annual Meeting of the American Association for Cancer Research in March, 2001 (abstract attached).

We have not yet characterized the size of the protein identified on PC-3 cells, either by immunoprecipitation or by Western blotting although we have been able to demonstrate that our anti-SIRP mAb can immunoprecipitate and blot SIRP α proteins of the correct molecular weight from CHO cells transfected with SIRP α . We are currently performing

these experiments using the prostate cell lines. Transcripts for SIRPs have been identified in PC-3 cells by dot blotting of RNA, but we have not yet completed analysis of transcript size by Northern blotting (Objective 1).

We have recently performed analysis of SIRP expression on human peripheral blood lymphocytes using the anti-SIRP mAb that we generated. These studies demonstrate that our antibodies stain not only human macrophages, as expected, but also show some binding to T and B cells (but not NK cells), which are not stained by previous anti-SIRP antibodies. We are currently investigating whether these T and B cells express member or the SIRP family or a cross-reacting molecule.

To obtain full-length cDNA clones (objectives 2 and 3), we have begun by pursuing hybridization cloning of cDNAs from PC-3 cells. These studies have been advanced by a collaboration with Drs. Shutsung Liao and John Kokontis at the University of Chicago, who have provided us with a cDNA library created from PC-3 cells. The first screen, using the SIRP α 1 probe obtained by PCR, identified only incomplete cDNA clones, so we are currently completing a second hybridization screen.

For studies of overexpressed SIRP α 1 in prostate cancer cells (Objective 4), we have succeeded in creating stable transfectants in PC-3 cells that overexpress either FLAG-tagged SIRP α 1 or SIRP β 1. We are now initiating studies of the effects of EGF on the growth of these cells compared to wild-type PC-3 cells. These studies will include examination of the effect of EGF on the phosphorylation of SIRPs in these cells and of associated proteins obtained by co-immunoprecipitation (Objective 5).

KEY RESEARCH ACCOMPLISHMENTS

1. The production of monoclonal antibodies to SIRPs.
2. The use of monoclonal antibodies to confirm the expression of SIRPs on prostate cancer cells.
3. Stable overexpression of SIRP α 1 and of SIRP β 1 in PC-3 prostate cancer cells.

REPORTABLE OUTCOMES

SIRPs are expressed on prostate cancer cell lines as assessed both by PCR and by fluorescence analysis (abstract attached)

CONCLUSIONS

Our findings with fluorescence analysis of prostate cancer cell lines confirm our initial observations by PCR that SIRPs are expressed on prostate cancer cell lines. Our stable transfectants of PC-3 prostate cancer cells, in which both SIRP α 1 and SIRP β 1 are independently overexpressed, will permit us to test the effect of these SIRPs on the response by PC-3 cells to epidermal growth factor, an autocrine regulator of the growth prostate cancer cells. Our collaboration with investigators at the University of Chicago will expedite the molecular cloning of SIRP cDNAs from PC-3 and LNCaP prostate cancer cells. Based on our progress to date, we expect to accomplish all of our objectives within the time frame of the grant.

Our studies under the DoD grant regard the use of prostate cancer cell lines to study the expression of SIRPs and their effect on the growth of prostate cancer cells. Our results to

date confirm the feasibility of this model and provide new tools with which to examine it (monoclonal antibodies to SIRPs, SIRP-transfected prostate cancer cells). Our findings also raise the issue of whether SIRPs are present in freshly prepared prostate cancer cells. We have initiated a collaboration for such studies with Dr. Peter Carroll, Chief of Urology at UCSF, and we will seek funding through the UCSD Cancer Center for a pilot study in this area.

REFERENCES

None

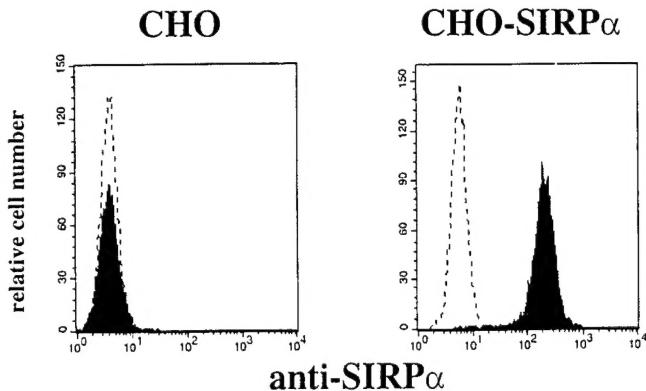


Figure 1. A monoclonal antibody (WES42) generated against SIRP α 1 stains Chinese Hamster Ovary (CHO) cells transfected with the cDNA for SIRP α 1 (right panel, solid histogram). The dotted histogram represents staining of cells by FITC-conjugated goat anti-mouse Ig alone.

To generate this and other anti-SIRP monoclonal antibodies, the cDNA for SIRP α 1 was first 'tagged' by placing a known epitope (FLAG) at the N-terminus, by use of recombinant cDNA. Antibody to FLAG was then used to identify expression of SIRP α 1 on CHO cells transfected with this construct. Stable transfectants were used to immunize BALB/c mice. Following fusion of spleen cells with Sp2/0 cells, supernatants were screened for binding to 293T cells transiently transfected with the same construct. Binding to FLAG was excluded by screening against FLAG expressed on a different protein, and binding to other determinants on CHO was excluded by staining untransfected cells, as shown in the figure (left panel).

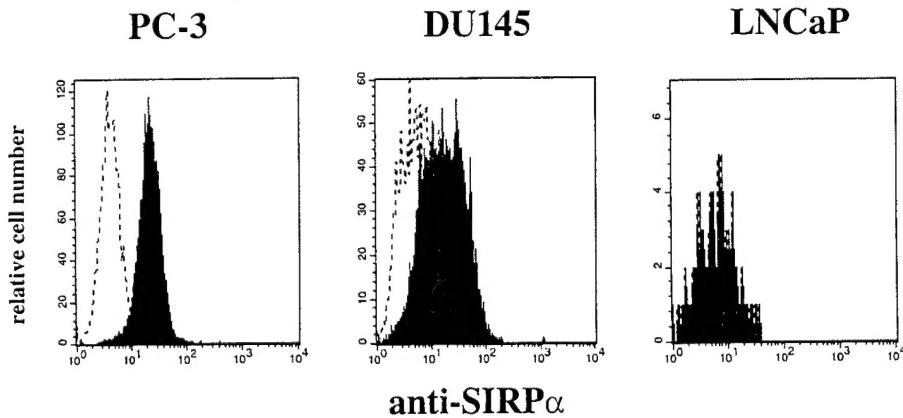


Figure 2. Monoclonal antibody to SIRP α 1 demonstrates the expression of SIRPs on prostate cancer cell lines. Prostate cancer cells were incubated with supernatants from hybridoma WES42 and then stained with FITC-conjugated goat anti-mouse Ig (solid histograms). The dotted histogram shows staining of cells by FITC-conjugated goat anti-mouse Ig alone. SIRPs are expressed on the surface of PC-3 cells and on DU-145 cells but could not be detected on LNCaP cells. Similar results were obtained with another anti-SIRP α 1 mAb (WES6) and with a monoclonal antibody to bovine SIRPs, which cross reacts with human SIRPs (IL-A24, from Dr. Nial MacHugh, Nairobi) (results not shown).

APPENDIX 2

Abstract accepted for presentation at the annual meeting of the American Association for Cancer Research (AACR), New Orleans, March 2001:

Prostate cancer cell lines express signal regulatory proteins (SIRPs). Nakamura MC, Spusta SC, Niemi EC, Chew K, MacHugh ND, Seaman WE. San Francisco VA Medical Center, University of California San Francisco, and International Livestock Research Institute, Nairobi.

The signal regulatory proteins (SIRPs, also known as SHPS-1, OX41, BIT, p84, MFR, and MyD1) are a family of immunoglobulin-like cell-surface receptors. There are two types of SIRPs, α and β . SIRP α is expressed on macrophages, dendritic cells, granulocytes, and certain cerebellar neurons. Its cytoplasmic domain can bind the tyrosine phosphatases SHP-1 and SHP-2. SIRP α appears to play an important role in the regulation of cell adhesion and the response to growth factors, including epidermal growth factor and growth hormone. The extracellular domain of SIRP α interacts with integrin-associated protein (CD47), which is expressed on a variety of cell types, including hematopoietic cells. On T lymphocytes, CD47 provides a costimulus for cell activation, while ligation of CD47 on dendritic cells inhibits their phenotypic and functional maturation. SIRP β is expressed on myeloid cells and delivers activation signals through an adaptor molecule, DAP12.

We here report that SIRPs are expressed on the prostate cancer cell line PC-3 and, to a lesser extent, on the prostate cancer cell lines DU-145 and LNCaP. The expression of SIRPs was first detected by RT-PCR from tumor cell RNA, by using primers common to both SIRP α and SIRP β . The primers spanned ~800 base pairs in the extracellular domain. A band of appropriate size was amplified from all three prostate cancer cell lines; it was brightest from PC-3 cells, lower from DU-145 cells, and only faint from LNCaP cells. Sequencing of the band from PC-3 cells confirmed that the sequence was from SIRP α . We next derived monoclonal antibodies reactive with both SIRP α and SIRP β and used these to confirm the expression of SIRPs on the surface of PC-3 cells and, to a lesser extent, on DU-145 cells. Expression of SIRPs on LNCaP cells was not detectable in this assay. A similar staining pattern was seen with a monoclonal antibody to bovine SIRPs (IL-A24), which cross-reacts with human SIRPs. The malignant potential of prostate cancer cells is associated with their response to growth factors and with their capacity to adhere to extracellular matrix. It may also depend on the host immune response, including T cells and dendritic cells. The unexpected finding of SIRPs on prostate cancer cells is relevant to each of these areas.